XIII_1402

Ovidius University Annals of Chemistry Volume 16, Number 1, 2005

Determination of ciprofloxacin in human plasma by high-performance liquid chromatography

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Abstract. A high-performance liquid chromatographic method has been developed for the determination of ciprofloxacin in human plasma. Effect of organic modifiers on the retention was investigated. Ciprofloxacin was determined using one isocratic elution of plasma supernatant after the precipitation of proteins. The chromatographic column was 5 μm Hibar Lichrospher 100 RP 8, the mobile phase being acetonitrile (0.5 %, *V/V*) and triethylamin (pH to 2.5 adjusted with H_3PO_4) (15:85, *V/V*). Analysis was run at a flow-rate of 1.2 ml min^{-1} and at a detection wavelength of 280 nm using DAD detector. The calibration curve was linear in the concentration range of 0.2-4.0 μg ml^{-1}.

Keywords: ciprofloxacin, determination, human plasma, high-performance liquid chromatography.

1. Introduction

The more recent introduction of ciprofloxacin represents a particularly important therapeutic advance, since this agent has broad antimicrobial activity and is effective after oral administration for the treatment of a wide variety of infection diseases [1]. There are several investigations concerning the determination of ciprofloxacin in pharmaceutical preparations by high performance liquid chromatography (HPLC) [2].

A number of assay methods for ciprofloxacin in biological fluids have been reported [3-10]. Many authors have performed the determination of ciprofloxacin in blood plasma or serum after protein precipitation [3-5]. These methods yielding poor separation of ciprofloxacin from the blood plasma and serum endogenous interferences and gave relatively low recoveries. The disadvantage of liquid-liquid extraction with different solvent [6, 7] is that they involve several steps and takes time. Other investigators have performed the separation of ciprofloxacin from plasma [8, 10] with solid-phase extraction on different cartridges (C_8 and C_{18}-bonded silica). The methods using solid-phase extraction technique are sensitive but needs internal standard and therefore they are more complicated for the analysis.

In our study we made a modification of HPLC method for determination of ciprofloxacin and ciprofloxacin metabolites in body fluids with fluorescent detection proposed by Krol et al. [9]. This procedure was used for the extraction of ciprofloxacin from plasma samples, before its determination with HPLC using UV detection. For the purpose of minimizing the variability caused by sample pretreatment we suggest a method of internal standardization for the quantification of these drugs. The method was first developed for the separation of and determination of ciprofloxacin by optimizing the experimental parameters. Then the method was validated by evaluating recovery, selectivity, linearity, precision and accuracy. Finally, the method was used for determination of ciprofloxacin plasma samples obtained from healthy volunteers.

2. Experimental

Materials

Ciprofloxacin was kindly supplied by Morepen Laboratories Limited (India). Internal standard of ofloxacin was supplied by Selectchemie AG, (Switzerland). HPLC grade acetonitrile and methanol were purchased from Across Organics (Belgium). Triethylamine (TEA), *o*-phosphoric acid,

ISSN-1223-7221 © 2005 Ovidius University Press

potassium dihydrogen phosphate and trichloroacetic acid were obtained from Merck (Germany).

Instrumentation

HPLC was performed using a Perkin-Elmer liquid chromatography system consisting of a pump PE LC series 200, autosampler PE LC ISS Series 200, diode array detector PE LC 235 C and column oven PE model 101. The chromatographic system is controlled by software package Turbochrom Version 4.1. plus and UV-spectrometric data are produced by program TurboScan Version 2.0.

A set of column packing including C8, C18 and RP-select B with different lengths and particle sizes were tested and the final choice of the stationary phase giving satisfying resolution and run time was a Hibar Lichrospher 100 RP 8, 250 x 4 mm I.D. (5 μm, particle size), protected by a guard column Lichrospher 100 RP 8, 4 x 4 mm (5 μm). A flow-rate of 1.2 ml min^{-1} was used for all separations of ciprofloxacin and internal standard with UV detection at 280 nm. A column temperature of 25 °C was used with an injection volume of 120 μl.

Preparation of standards and plasma samples

Stock solutions of ciprofloxacin and internal standard (ofloxacin) were prepared at concentration 1000 μg ml^{-1}. These solutions were prepared monthly and stored at 4 °C. No change in stability over the period of 1 month was observed. The working solutions were prepared by diluting appropriate portions of these solutions with distilled water.

Human plasma was prepared from heparinized whole blood samples. Blood samples were collected from healthy volunteers who later participated in a bioequivalence study, and stored at –20 °C. After thawing, samples were spiked daily with working solutions of ciprofloxacin and internal standard. All plasma samples were prepared by taking of 0.5 ml aliquot of plasma sample and diluted with 0.1 ml of 0.1 mol l^{-1} phosphoric acid and 0.3 ml of 5.0 mol l^{-1} trichloroacetic acid-acetonitrile (1:1, *V/V*) solution. The mixture was vortexed and diluted again with 0.1 ml of acetonitrile and 0.3 ml of water. The final solution was vortexed and centrifuged for 10 min at 10000 g. The aliquot of supernatant was transferred into a glass autosampler vial for HPLC analysis.

Calibration curves

Calibration curve was constructed with six blank plasma samples spiked with appropriate amounts of the standard solutions. The calibration range was 0.2-4.0 μg ciprofloxacin per ml of plasma. The standard samples were prepared according to the procedure as unknown samples. The calibration curve was obtained by plotting the peak height ratio of ciprofloxacin to internal standard versus its concentration in μg/ml. The regression equations were calculated by the least-squares method. Quality control samples were prepared at low, medium and high levels in the same way as plasma samples for calibration.

3. Results and Discussions

Method development

Several HPLC method variables with respect to their effect on the separation of ciprofloxacin and internal standard from the matrix were investigated (different pH values in combination with different organic modifiers). The amount of organic modifier present in the mobile phase will influence on analytes that are retained predominantly by adsorption onto the stationary phase. Fig. 1 shows the results that were obtained over an acetonitrile range of 10 % to 20 % in the mobile phase (0.5 %, *V/V*, triethyl amin, pH 2.5 adjusted with H_3PO_4). This data was used to determine an optimal amount of organic modifier that should be used for the separation of ciprofloxacin. The best results (good separation between three peaks of ciprofloxacin and internal standard, ofloxacin, short time of analysis) can be obtained using mobile phase with 15 % of acetonitrile.

Additional study was also done to check the effect that percentage of TEA in mobile phase had on analytes retention. Fig. 2 shows the results that were obtained over a percentage of TEA range of 0.0 % to 1.0 % in the mobile phase (TEA, pH 2.5 adjusted with H_3PO_4 and 15 % acetonitrile). As the percentage of TEA in mobile phase was increased, ciprofloxacin and internal standard decreased in retention. Also, before the addition of TEA, investigated drugs showed peak tailing. The peak tailing may be attributed to hydrogen bonding between the free silanols of the stationary phase and the amino groups on quinolones. Actually, TEA is acting like an organic modifier. In the other hand,

the mobile phases that containing TEA had much better peaks shape. This improved peaks shape may be due to the TEA blocking the fixed silanol sites. Also, adsorbed TEA prevented hydrogen bonding with the amino groups on investigated quinolones.

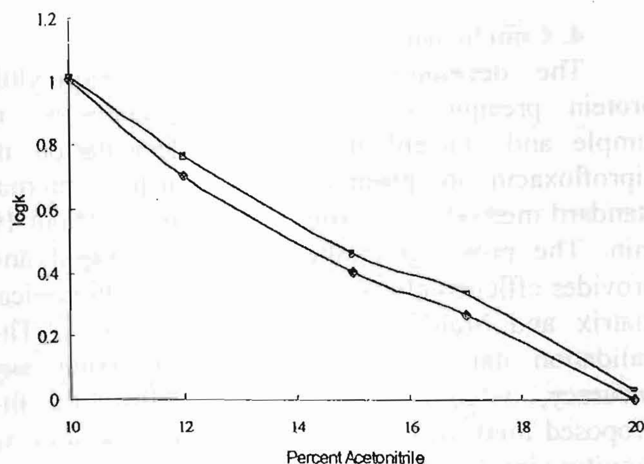

Fig. 1. The effect of acetonitrile on ciprofloxacin retention (■-ofloxacin; ◆- internal standard)

From these data it was determined that mobile phase consisted of 0.5 % TEA, pH 2.5 adjusted with H_3PO_4 and 15 % acetonitrile would provide good retention for ciprofloxacin (7.5 min) and internal standard as well as an acceptable runtime of less than 10 min for the separation.

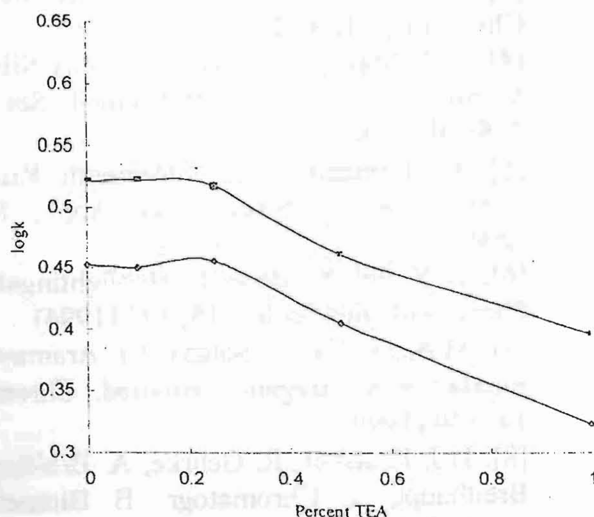

Fig. 2. The effect of TEA concentration on ciprofloxacin retention (■-ofloxacin; ◆-internal standard)

In addition, different reagents (acetonitrile, perchloric acid, trichloroacetic acid) were used for protein precipitation in order to obtain satisfactory values for recovery of ciprofloxacin and internal standard. Plasma sample were treated with equal volume of acetonitrile or 6% trichloroacetic acid. For precipitation of plasma protein, in 0.25 ml spiked plasma samples, a 0.05 ml of 20 % perchloric acid were added (procedure 3). The extraction recoveries were calculated by comparing the peak height of ciprofloxacin and internal standard obtained for two concentration levels (n=3 for each level for investigated drugs, n=6 for internal standard) and those resulting from the direct injection (n=3, working solutions) of the theoretical amount of drugs (=100 % recovery). Results of this investigation are presented in Table 1.

Table 1. Absolute recoveries of ciprofloxacin from spiked plasma samples

Procedure	Mean recovery (%)		
	c(cipr.) = 1 μg ml^{-1}	c(cipr.) = 2 μg ml^{-1}	c(int. stand.) = 2 μg ml^{-1}
1	10.73	11.69	14.14
2	62.13	67.39	74.20
3	64.36	67.44	73.63
4	96.16	95.26	93.87

As can be seen, the satisfactory values for recovery of ciprofloxacin and internal standard were obtained when plasma samples were prepared according to previously reported method for determination of ciprofloxacin in body fluids by Krol et al. [9] (procedure 4 in Table 1). Under the chromatographic conditions described, ciprofloxacin peak was well resolved. Endogenous plasma components did not give any interfering peaks. The developed HPLC method was used for analysis of plasma samples from healthy volunteers after oral administration of ciprofloxacin.

Method validation

Linearity, precision and accuracy

Linearity was tested in three different days at five concentration points ranged from 0.2-4.0 μg ml^{-1} of ciprofloxacin in plasma samples. The correlation coefficient was 0.9969. In one day and in 3 different days, spiked samples from each concentration used for construction of calibration curve were prepared in triplicate and analyzed. Then, the corresponding coefficients of variation were calculated. The intra- and inter-day variations of the method throughout

the linear range of concentrations (Table 2) indicate a considerable degree of precision and reproducibility for the method both during one analytical run and between different runs.

Table 2. Intra-and inter-day precision data

Precision data	Nominal concentration/μg ml⁻¹				
	0.2	0.5	1.0	2.0	4.0
Intra-day (n=3)					
c(cipr.)/μg ml⁻¹	0.22	0.54	1.08	1.87	4.14
RSD, %	2.93	4.55	3.23	4.83	3.71
Inter-day (n=9)					
c(cipr.)/ μg ml⁻¹	0.21	0.53	1.08	1.85	4.07
RSD, %	5.07	3.80	4.51	3.97	2.88

Intra- and inter-day accuracy was determined by measuring plasma quality control samples at low, middle and high concentration levels of ciprofloxacin. An indication of accuracy was based on the calculation of the relative error of the mean observed concentration as compared to the nominal concentration (Table 3). Relative error at concentrations studied for ciprofloxacin is less than 7.5 % and it is obvious that the method is remarkably accurate which ensures obtaining of reliable results.

The limit of quantification was defined as the lowest amount detectable with a precision <15 % (n=5) and an accuracy of ±15 % (n=5). The limits of quantification was found to be 0.2 μg·ml⁻¹.

Table 3. Intra-and inter-day accuracy data

Accuracy data	Nominal concentration/μg ml⁻¹		
	0.3	0.8	1.3
Intra-day (n=3)			
c(ciprofloxacin) /μg ml⁻¹	0.31	0.83	1.29
Relative error (%)	3.33	3.75	-0.77
Inter-day (n=9)			
c(ciprofloxacin)/μg ml⁻¹	0.32	0.84	1.27
Relative error (%)	6.67	5.0	-2.31

Stability of ciprofloxacin in plasma samples

Stability of ciprofloxacin in plasma was investigated using spiked samples at two different concentration levels prepared in duplicate. Spiked samples were analysed after different storage conditions: immediately, after staying in an autosampler for 2, 12 and 24 h, after one and two freeze/thaw cycles and after two weeks stored at –20 °C. The results from this investigation show that ciprofloxacin added to plasma is stable in the different storage conditions.

4. Conclusions

The developed HPLC method employing protein precipitation for sample preparation is simple and convenient for the determination of ciprofloxacin in plasma samples using internal standard method. The typical assay time is about 10 min. The proposed method is simply, rapid and provides efficient clean up of the complex biological matrix and high recovery of ciprofloxacin. The validation data demonstrate good precision and accuracy, which proves the reliability of the proposed method. Also, the method can be used to monitor ciprofloxacin levels in clinical samples.

5. References

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